Anticoagulant activity of a synthetic heparinoid in relation to molecular weight and *N*-sulfate content

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Addition of chlorosulfonyl isocyanate to C=C bonds in *cis*-1,4-polyisoprene and reaction of the adduct with NaOH resulted in the formation of a water-soluble polyelectrolyte with *N*-sulfate and carboxylate

groups. The polyelectrolyte showed anticoagulant activity and it was found, just as with heparin, that the activity was related to molecular weight and *N*-sulfate content.

INTRODUCTION

Modification of polymers has often been realized by chemical transformation of functional groups and in this way polymers with specific properties could be obtained.¹

In previous publications we reported about the modification of unsaturated polymers and the synthesis of water-soluble polyelectrolytes with anticoagulant activity.²⁻⁶ The anticoagulant activity, by measuring recalcification times, was about 1/20 of heparin using bovine plasma and about 1/40 of heparin using human plasma. One of the polyelectrolytes was derived from *cis*-1,4-polyisoprene in which the following structural units, apart from unreacted isoprene units, were present:

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It was found that this polyelectrolyte, just like heparin, acts as an anti-thrombin. There are reasons to assume that the presence of both *N*-sulfate and carboxylate groups is essential for the anticoagulant activity, indicating a further resemblance with heparin.⁵

The anticoagulant activity of heparin is related to the molecular weight and to the *N*-sulfate content.⁷⁻¹¹ This paper describes the effect of these parameters on the anticoagulant activity of polyelectrolytes, synthesized from *cis*-1,4-polyisoprene.

EXPERIMENTAL

Materials

Polyelectrolytes were synthesized according to a method described before.²⁻⁵ The starting material for the synthesis of two slightly different polyelectrolytes PLE-A and PLE-C was *cis*-1,4-polyisoprene (Cariflex IR 307, Shell). The *cis*-1,4-content of this polyisoprene was more than 96% and the molecular weight was $\bar{M}_n = 270.000$, $\bar{M}_w / \bar{M}_n = 5.3$. For the synthesis of polyelectrolytes PLE-B, *cis*-1,4-polyisoprenes with lower molecular weights were used. The latter were prepared from isoprene according to a method given by Braun et al.¹² From NMR measurements a *cis*-1,4-content of 90%–95% could be concluded. Solutions of *cis*-1,4-polyisoprene in toluene were added to stirred solutions of chlorosulfonyl isocyanate (CSI) in the same solvent. Reactions were carried out at 20 °C (PLE-A and PLE-C) and at 0 °C (PLE-B) in a nitrogen atmosphere. For the synthesis of the polyelectrolytes of the PLE-B series always 5 g of the polyisoprene and 35 g CSI was used. Polyelectrolytes were synthesized from the CSI-adducts by hydrolysis with a sodium hydroxide solution at 100 °C. For the low molecular weight polyelectrolytes (PLE-B 1–4) it was possible to carry out the hydrolysis at 20 °C. The polyelectrolytes were purified by dialysis against distilled water for three days, followed by freeze-drying of the solutions. For the dialysis of aqueous solutions of low molecular weight polyelectrolytes (PLE-B 1–4) benzoylated dialysis tubing (Sigma D 7884) was used, whereas solutions of high molecular weight polyelectrolytes were dialyzed with normal cellulose tubing (Cenco).

Fractionation of PLE-A was accomplished using ethanol–water mixtures analogous to a procedure described by Lasker and Stivala.⁷ The first fraction was soluble in an ethanol–water mixture with a volume ratio of 70/30. Extraction was continued by increasing the water content in steps of 2%. The last fraction dissolved in a 62/38 ethanol–water mixture. The solvents were partly removed under reduced pressure and the fractions were obtained by freeze-drying of the remaining solutions.

For the desulfation experiments portions of PLE-C were brought in 0.1M HCl at 20 °C for different periods of time. Subsequently sodium hydroxide was added until pH > 7, and the solutions were dialyzed against distilled water for three days. The reaction products were isolated by evaporation of the water.

Methods

Molecular weights ($\overline{M}_n$ and $\overline{M}_w$) were determined by GPC on a Waters 4-column instrument equipped with a differential refractometer as a detector. The column length was 4 ft per column, O.D. $\frac{3}{8}$ in. and the porosity sequence of the crosslinked polystyrene packing (Styra gel) 10^5 – 3×10^4 – 10^3 –250 Å. The eluant was tetrahydrofuran, the flow rate 1 mL/min⁻¹, the temperature 30 °C.

The relation between GPC-elution volume and $\overline{M}_n$ was established by determination of $\overline{M}_n$ for several samples by means of a Hallikainen automatic membrane osmometer. The membrane was Sartorius SM 11539 (ultracella "allerfeinst"). The relation between $\overline{M}_w$ and the GPC-elution volume was determined by measuring $\overline{M}_w$ for several samples by means of a Fica 50 light scattering photometer. An empirical correction for peak-broadening (determined for a series of standard polystyrenes) was applied:

$$(\overline{M}_n)_{\text{GPC}} = 1.20 \times (\overline{M}_n)_{\text{true}}, \quad (\overline{M}_w)_{\text{GPC}} = 1.00 \times (\overline{M}_w)_{\text{true}}$$

Viscosity measurements of the polyelectrolytes were performed at 25 °C in an Ubbelohde viscometer with capillary length 12.8 cm and capillary diameter 0.4 mm. In 0.1M NaCl solutions straight lines were obtained when η_{spec}/c was plotted against c (usually 5 concentrations; 0.1 and 0.5 g/100 mL as limits).

Elemental analyses were performed by the Organic Chemical Institute TNO, Utrecht, The Netherlands. The absolute error in the weight percentages of the elements was 0.2%.

For the determination of the anticoagulant activity recalcification times and/or thrombin times of citrated human plasma (Red Cross, Groningen) were measured. The plasma, stored at –28 °C, was thawed a few days before the experiments and spread over 5–20 mL portions (polypropylene tubes) and stored at –28 °C again. Immediately before the experiments the plasma was thawed and stored in an icebath. Prior to use the pH was adjusted to 7.4 with 0.15M HCl or 0.15M NaOH.

Recalcification times

Recalcification times were determined at 37 °C in Pyrex tubes with an O.D. of 1.0 cm. The coagulation of the plasma was followed in an optical way, using a Vitatron colorimeter, model UC 200 S equipped with a special thermostated vessel holder for the test tube.³ Polyelectrolyte stock solutions of 12 mg/mL water (pH 7.4) were used for all the recalcification experiments; 40 µL of this solution was added to 1 mL of plasma. After 5 min of incubation recalcification was carried out by the addition of 60–80 µL 0.3M CaCl₂ (pH 7.4). The recalcification times of plasma in the presence of polyelectrolyte are denoted by $t_{\text{PLE-C}}$ (standard sample) and $t_{\text{PLE-C X}}$ (test sample), the recalcification time of plasma in glass by t_g . The relative anticoagulant activities of the polyelectrolytes PLE-C X are defined as

$$(t_{\text{PLE-C X}} - t_g)/(t_{\text{PLE-C}} - t_g) \times 100\%$$

Thrombin times

Thrombin times were measured according to the method of Studer and Winterstein.¹³ Polyelectrolyte stock solutions of 0.2 mg/mL water (pH 7.4) were used for all the experiments, 50 μ L of the polyelectrolyte solution was added to 950 μ L of plasma and this mixture was prewarmed in a Clot-timer (Heller laboratories) during about 5 min. After incubation 100 μ L of the mixture was added to 200 μ L of a prewarmed thrombin solution (15 NIH/mL water) and the thrombin time was measured. Using heparin calibration curves the anticoagulant activity of the polyelectrolytes was expressed in International Heparin Units (IU/mg).

RESULTS AND DISCUSSION

Cis-1,4-polyisoprene was modified as shown in Scheme 1. The double bonds reacted with CSI to an extent of 60–70 mol %, leading to a modified polymer with structures (a) and (b).^{5,6}

SCHEME 1

Structural units in modified *cis*-1,4-polyisoprene.

Reaction of the polymer-CSI adduct with NaOH resulted in the formation of

a water-soluble polyelectrolyte with structural units (a_1) and (b_1), apart from unreacted isoprene units.

The polyelectrolyte showed anticoagulant activity and in order to investigate the effect of the molecular weight on the activity, a polyelectrolyte batch was fractionated with ethanol-water mixtures, analogous to the method described by Lasker and Stivala⁷ for heparin. They isolated heparin fractions differing in molecular weight as well as in anticoagulant activity.

The results of the fractionation of PLE-A are shown in Tables I and II. From these data it can be seen that fractionation of the polyelectrolyte PLE-A resulted in polyelectrolytes which differed in *N*-sulfate content, intrinsic viscosity and anticoagulant activity. It is not possible, however, to conclude from these results a relationship between the *N*-sulfate content or the molecular weight and the anticoagulant activity. The differences between the intrinsic viscosities of the fractions 2, 3, 4, and 5 might be attributed rather to differences in *N*-sulfate content than to differences in chain length (molecular weight). The differences between the intrinsic viscosities of fraction 1 and the other fractions on the other hand, cannot only be explained by differences in *N*-sulfate content.

The effect of the molecular weight can be studied, however, by investigation of polyelectrolytes differing in molecular weight, but with the same *N*-sulfate

TABLE I
Analytical Data of the Fractionation Products of Polyelectrolyte PLE-A

Polyelectrolyte	Recovery (%)	N (%)	S ^a (%)	C/N ^b	N/S ^c	Mol % Addition ^d
PLE-A 1	1.8	5.3	6.2	8.7	1.9	65.1
PLE-A 2	38	5.5	8.3	8.4	1.5	67.9
PLE-A 3	26	5.2	8.4	8.9	1.4	63.2
PLE-A 4	23	5.2	9.0	8.6	1.3	66.0
PLE-A 5	7.5	5.1	9.5	8.4	1.2	67.6

^a % S $\times 10/32$ = mmol *N*-sulfate/g.

^b ± 0.3 .

^c ± 0.1 .

^d Calculated on % N.

TABLE II
N-sulfate Content, Intrinsic Viscosity and Anticoagulant Activity of the Fractionation Products of Polyelectrolyte PLE-A

Polyelectrolyte	mmol <i>N</i> -sulfate/g ^a	$[\eta]$ (100 mL/g)	Anticoagulant Activity (IU/mg) ^b
PLE-A 1	1.94	0.17	60
PLE-A 2	2.60	0.70	147
PLE-A 3	2.63	0.76	162
PLE-A 4	2.82	0.82	196
PLE-A 5	2.97	0.89	206

^a ± 0.06 . ^b As determined by thrombin time assay in conjuncture with heparin calibration curve.

content. Therefore polyisoprenes have been synthesized with different molecular weights and it was assumed that the reaction of CSI with the polyisoprenes should result in polyelectrolytes with about the same *N*-sulfate content, but with different molecular weights.

The addition reactions were carried out at 0 °C, because at this temperature less side reactions occur, compared with the addition reaction at room temperature.⁶ The results of the elemental analysis, viscosity measurements, and of the determination of the anticoagulant activities are shown in Tables III and IV and in Figure 1. It can be seen from these Tables that the *N*-sulfate content of the polyelectrolytes was approximately the same, whereas the polyelectrolytes differed markedly in intrinsic viscosity and anticoagulant activity (thrombin times).

These results indicate a relationship between the molecular weight and the anticoagulant activity of the polyelectrolytes. A sharp decrease of the activity was found below a certain molecular weight, corresponding with an intrinsic viscosity of about 0.15.

In this respect we see a striking resemblance with heparin, because also for heparin a lower anticoagulant activity was only found below a certain molecular weight.^{8,9}

TABLE III
Conversion of *cis*-1,4-Polyisoprenes into Polyelectrolytes PLE-B

Cis-1,4-polyisoprene		Polyelectrolyte				
$\bar{M}_n$	$\bar{M}_w$	PLE-B	N (%)	S (%)	Mol % ^a Addition	mmol ^b N-sulfate/g
1800	2000	PLE-B 1	5.7	9.5	72.0	2.97
2000	2100	PLE-B 2	5.5	9.7	68.6	3.03
3600	3800	PLE-B 3	5.3	9.8	69.0	3.07
6700	7700	PLE-B 4	5.2	9.6	64.6	3.00
7000	9000	PLE-B 5	5.3	9.2	64.6	2.88
14,000	18,000	PLE-B 6	5.1	10.2	65.2	3.19
25,000	35,000	PLE-B 7	5.2	10.0	66.8	3.13
51,000	71,000	PLE-B 8	4.9	9.6	60.8	3.00

^a Calculated on % N. ^b ± 0.06 .

TABLE IV
Intrinsic Viscosities and Thrombin Times of the Polyelectrolytes PLE-B

Polyelectrolyte	$[\eta]$ (100 mL/g)	Thrombin Time ^a (s)	<i>n</i>	S.D.
PLE-B 1	0.06	7.2	16	0.4
PLE-B 2	0.08	7.8	21	0.6
PLE-B 3	0.11	10.5	16	0.4
PLE-B 4	0.15	13.1	20	1.6
PLE-B 5	0.15	13.0	24	1.0
PLE-B 6	0.30	15.9	41	2.4
PLE-B 7	0.55	15.9	31	2.5
PLE-B 8	1.15	18.4	25	2.1

^a For heparin (Diosynth, 147.1 IU/mg) a mean thrombin time of 17.1 s was found (*n* = 60, S.D. = 1.4). In absence of heparin a mean thrombin time of 7.0 s was found (*n* = 36, S.D. = 0.7).

Figure 1. Thrombin time of human plasma in the presence of polyelectrolytes PLE-B as a function of the intrinsic viscosity. Bars indicate standard deviation.

In order to study the influence of the *N*-sulfate content on the anticoagulant activity of the polyelectrolytes, portions of PLE-C were brought in an acidic medium for different periods of time. In an acidic medium rupture of N-S bonds in the *N*-sulfate groups in structures (a₁) occurs.^{5,14} The influence of N-S rupture on the anticoagulant activity can be seen from Tables V and VI.

The observed decrease of the anticoagulant activity is assumed to be due to cleavage of N-S bonds, indicating that *N*-sulfate groups are necessary for the activity.

TABLE V
Influence of N-S Rupture on the Anticoagulant Activity of Polyelectrolytes PLE-C

Reaction time in 0.1M HCl ^a at 20 °C (h)	Polyelectrolyte	C/N ^b	mmol ^c N-sulfate/g	Relative Anticoagulant Activity ^d (%)
0	PLE-C	8.0	3.10	100
24	PLE-C 1	8.3	2.85	75
68	PLE-C 2	8.2	2.60	23
138	PLE-C 3	8.0	2.47	4

^a 1 g polyelectrolyte in 50 mL 0.1 M HCl.

^b ± 0.3 .

^c ± 0.06 .

^d Calculated from recalcification times of human plasma. $t_{\text{PLE-C}} = 10\text{--}12$ min ($c = 0.046$ mg PLE-C/mL plasma); $t_g = 4\text{--}5$ min.

TABLE VI
Thrombin Times and Intrinsic Viscosity of Polyelectrolyte PLE-C after treatment with 0.1M HCl at 20 °C

Polyelectrolyte ^a	N/S ^b	$[\eta]^c$ (100 mL/g)	Thrombin Time ^d (s)	<i>n</i>	S.D.
PLE-C	1.4	0.65	19.0	8	1.0
PLE-C 1	1.5	0.45	15.9	8	0.5
PLE-C 3	1.9	0.35	11.4	8	0.6

^a No data for PLE-C 2 are given, because only a limited amount was available.

^b ± 0.1 .

^c Measured in 0.1M NaCl, 25 °C.

^d For heparin (Diosynth, 147.1 IU/mg) a mean thrombin time of 17.1 s was found ($n = 60$, SD = 1.4). In absence of heparin a mean thrombin time of 7.0 s was found ($n = 36$, SD = 0.7).

In this respect a further resemblance with heparin is observed, because *N*-sulfate groups have also been reported to be essential for the anticoagulant activity of heparin.⁷⁻¹⁰

It must be mentioned that besides an increase of the N/S ratio after treatment with 0.1M HCl there is also a small decrease of the intrinsic viscosity (probably mainly due to loss of sulfate groups). The decrease of the anticoagulant activity cannot be explained, however, by the small decrease of the intrinsic viscosity (see also Fig. 1).

No data are available yet about the distribution of *N*-sulfate groups in the polyelectrolytes. This effect should be taken into account, because it has been reported that the distribution of *N*-sulfate groups is a factor in the activity of heparin.^{8,9}

It can be concluded from the present results, however, that the molecular weight and the *N*-sulfate content of the polyelectrolytes are important parameters for the anticoagulant activity.

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References

1. P. Rempp, "Recent Results on Chemical Modification of Polymers," *Pure Appl. Chem.*, **46**, 9 (1976).
2. L. van der Does, J. Hofman, and T. E. C. van Utteren, "Reaction of *N*-chlorosulfonylisocyanate with Unsaturated Polymers. Route to a Synthesis of Polyampholytes," *J. Polym. Sci. Polym. Lett. Ed.*, **11**, 169 (1973).
3. T. Beugeling, L. van der Does, A. Bantjes, and W. L. Sederel, "Antithrombin Activity of a Polyelectrolyte Synthesized from *cis*-1,4-Polyisoprene," *J. Biomed. Mater. Res.*, **8**, 375 (1974).
4. T. Beugeling, L. van der Does, B. V. Rejda, and A. Bantjes, "Antithrombogenic Polymers synthesized from Polyisoprenes," in *Biocompatibility of Implant Materials*, D. Williams, Ed., Sector, London, 1976, p. 187.
5. L. van der Does, T. Beugeling, P. E. Froehling, and A. Bantjes, "Synthetic Polymers with Anticoagulant Activity," *J. Polym. Sci. Polym. Symp.*, **66**, 337 (1979).

6. L. van der Does, J. F. van Duijl, L. C. Sederel, and A. Bantjes, "Modified Polymers from Cis-1,4-Polyisoprene," *J. Polym. Sci. Polym. Lett. Ed.*, **18**, 53 (1980).
7. S. E. Lasker and S. S. Stivala, "Physicochemical Studies of Fractionated Bovine Heparin," *Arch. Biochem. Biophys.*, **115**, 360 (1966).
8. J. A. Cifonelli, "The Relationship of Molecular Weight and Sulfate Content and Distribution to Anticoagulant Activity of Heparin Preparations," *Carbohydr. Res.*, **37**, 145 (1974).
9. J. A. Cifonelli, "Relation of Chemical Structure of Heparin to its Anticoagulant Activity," *Adv. Exp. Med. Biol.*, **52**, 95 (1975).
10. M. W. Piepkorn, D. Lagunoff, and G. Schmer, "Heparin Binding to Antithrombin III: Variation in Binding Sites and Affinity," *Biochem. Biophys. Res. Commun.*, **85**, 851 (1978).
11. T. C. Laurent, A. Tengblad, L. Thunberg, M. Höök, and U. Lindahl, "The Molecular Weight Dependence of the Anticoagulant Activity of Heparin," *Biochem. J.*, **175**, 691 (1978).
12. D. Braun, H. Cherdron, and W. Kern, "Praktikum der Makromolekularen Organischen Chemie," Dr. Alfred Hüthig Verlag, Heidelberg, 1966.
13. A. Studer and A. Winterstein, "Probleme der Blutgerinnung," *Helv. Physiol. Acta.*, **9**, 6 (1951).
14. L. C. Sederel, L. van der Does, T. Beugeling, J. F. van Duijl, and A. Bantjes, "Anticoagulant Activity of Polyelectrolytes and their Behaviour in Surface Coatings," presented at the Third International Conference on Plastics in Medicine and Surgery of the Plastic and Rubber Institute, Enschede (the Netherlands), June 21-22, 1979.

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